EMBO reports

Non-canonical ATM/MRN activities temporally define the senescence secretory program

Nicolas Malaquin, Marc-Alexandre Olivier, Aurelie Martinez, Stéphanie Nadeau, Christina Sawchyn, Jean-Philippe Coppé, Guillaume Cardin, Frédérick Mallette, Judith Campisi and Francis Rodier

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1st Editorial Decision 8th May 2020

Dear Francis,

Thank you for the transfer of your manuscript with referee comments to EMBO reports. We would like to invite you to address the last concerns by referee 2 and to resubmit a final manuscript as soon as possible.

Please make sure that all statistical information are included in the figure legends, that the abstract is written in present tense, that the reference style is the numbered EMBO reports style (see our guide to authors), and please also submit a short 1-2 sentence summary of your findings and their significance along with 2-3 bullet points highlighting key results and a synopsis image that is 550 pixels wide x 200-400 pixels high.

The supplementary figures can be changed into Expanded View (EV) figures, or need to be moved into an Appendix file (see our guide to authors). We allow a maximum of 5 EV figures (plus EV tables) but if you cannot combine or reduce the Ev figures to 5 we can exceptionally also accept 6. The EV figure legends should be added to the main manuscript after the main figure legends. The EV figures should be uploaded as one file per figure.

I am looking forward to receiving a final manuscript as soon as possible.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors report that HDACi trigger senescence and SASP without markers of DDR activation and yet MRN and ATM knockdown reduce SASP while ATM kinase inhibitors and MRN inhibitors don't (or do it weakly).
However, MRN and ATM accumulate on chromatin in senescent cells and on them depends NFkB accumulation on chromatin.

How MRN and ATM are engaged in senescent cells in the absence of detectable DNA damage remains unclear. Similarly, their role in NF-KB activation is unprobed.

Overall the revised manuscript is now a coherent story that yet lacks the insights usually read in EMBO J.

Referee #2:

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1. In Fig1. authors clearly suggest that dynamics of SASP is different for NaB and XRA treated cells, with IL6 secretion being significantly accelerated in HDACi. Authors show that for HCA2-hT fibroblasts, peak of IL-6 secretion for NaB treated cells is 4-5 days, whereas for XRA-treated cells it is 10 days. Interestingly, level of IL6 decreases ~20 times from day 4-5 to day 8-9 for NaB treated cells (Fig. 1C, 1E). Do authors quantify the senescent cells at day 4-5 for NaB treated cells? If the level of senescent cells is decreasing between day 5 and 9 for NaB treated cells, how authors would explain it? How authors can verify that HDACi-induced SASP is not transient?
2. Point 2.2. shall be better addressed by authors. First of all, authors show in the last part of the paper, the binding of NF-kB to chromatin 2-3 days following NaB treatment. As authors explain, SASP requires activation of NF-kB pathway, involving NF-kB translocation to nucleus and binding to chromatin at SASP promoter sites. Authors could confirm by ATAC-seq, that p65 binds at SASP promoter sites in HDACi-induced SASP. In addition, authors could do co-culture experiment of NaB-treated cells with growing cells, and verify if cytokine secretion triggered by HDACi can induce senescence of growing cells in paracrine manner?
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4. Minor comment:
- Authors shall consider to be consistent with the figure units (unit of time), Fig 1E, 1F, 1G, 1H - units in days, Fig 1I units in hours.
- In the text, authors refer to Fig. 3G (no such figure).

Additional cross-comment referee #2:

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The observations from the reviewer concerning the difference in the secretion levels of Il-6 between figure 1C (10 days of NaB) and 1E (5 days of NaB) are interesting, it is something we did not emphasize correctly. In figure 1C, HCA2hT cells were treated long-term with 2mM of NaB while the treatment in figure 1E was shorter, but using 5mM, so the higher dose is apparently consistent with higher Il-6 levels (for longer term treatments we used lower dose to minimize cell stress as previously suggested (Ogryzko, Hirai et al., 1996). We notice that the description of these experimental conditions in the figure legends is confusing. We now clearly state that 2mM of NaB is used for the long-term treatment in figure 1B, C and D and that for all other experiments the dose of NaB is 5mM. Regarding the verification of whether senescent cells number decrease between days 3 and 9 during long-term 2mM NaB treatment, which would perhaps reveal an interesting observation with an early peak of senescence followed by a decline, the answer is that this does not happen. We have now added additional data to show a continual increase in senescent cells over time after treatment, and thus this result is consistent with the usual gradual accumulation of cells harboring senescence hallmarks (new FigS1A).

How authors can verify that HDACi-induced SASP is not transient?

This is a good question, although perhaps less important given we show above that the HDACi-SASP is likely dose-dependent and that senescence hallmarks measured gradually increase over time, they do not decrease. Also, the stability of the HDACi senescence phenotype when using the long-term treatment was previously tested by others when originally described (Ogryzko et al., 1996). Similarly, the SASP triggered by HDACi treatment is stable enough that co-injection of

HDACi-senescent fibroblast with cancer cells promote cancer cell proliferation in xenograft experiments (Paracrine effect;(Pazolli, Alspach et al., 2012)). Of note, to support the similarity of the DNA damage and HDACi SASP we performed antibody arrays with large-scale correlations and extensively used multiplex assays revealing the secretory profiles are matched. Thus, from the previous literature and this paper, we can assume that the HDACi-SASP is at least relatively stable and almost identical to other types of SASP previously described. We now mention this in the discussion

Furthermore, regarding stability, it could very well be that similarly to the reversibility of the p16-mediated senescence proliferation arrest (Beausejour, Krtolica et al., 2003, Narita, Nunez et al., 2003), the irreversibility of the HDACi-SASP (or any type of SASP) may take a long period of time to establish, or never fully establish. Along those lines, it is now known that previously described SASP induced by DNA damage or oncogenes or replication stress keeps evolving over time for at least many months, perhaps indefinitely (De Cecco, Ito et al., 2019, Ito, Teo et al., 2018, Martínez-Zamudio, Roux et al., 2019). Thus, to precisely answer this question, we could perform a long series of treatment and release sequences, of a-priori unknown duration, probably for at least 2-3 weeks followed by SASP assessments. Despite this comment being an interesting question, we would argue that reversibility (or not) of the HDACi-SASP is not part of the message we convey. Whether we find that this SASP is reversible (or not) will not change or strengthen the conclusions we draw. The key point that we are making is that overall the SASP program is activated rapidly in an ATM-dependent manner in the absence of DNA damage by HDACi, and that a similar phenotype take more time to occur in response to DNA damage, hence supporting the idea that the presence of direct DNA damage or classical DDR activation is not the most important source of SASP activation.

2. Point 2.2. shall be better addressed by authors. First of all, authors show in the last part of the paper, the binding of NF-kB to chromatin 2-3 days following NaB treatment. As authors explain, SASP requires activation of NF-kB pathway, involving NF-kB translocation to nucleus and binding to chromatin at SASP promoter sites. Authors could confirm by ATAC-seq, that p65 binds at SASP promoter sites in HDACi-induced SASP. In addition, authors could do co-culture experiment of NaB-treated cells with growing cells, and verify if cytokine secretion triggered by HDACi can induce senescence of growing cells in paracrine manner?

(from previous response: 2.2. It is possible that HDACi trigger cytokine secretion also in other contexts, unrelated to senescence. How it is possible to conclude that what HDACi triggers is SASP? Can HDACi trigger similar cytokine expression to senescent cells in vivo or in physiological context?

The reviewer is right that perhaps HDACi can trigger pro-inflammatory secretion without senescence in other context. Like all senescence hallmarks, the secretion of pro-inflammatory factors is not unique/specific to senescent cells. But taken together with other senescence hallmarks, SASP is used to define the senescence state. Here, we made sure to show that HDACi induces a full panel of senescence hallmarks (as others have already showed in similar contexts (Ogryzko et al., 1996, Pazolli et al., 2012). To our knowledge there are no other ways to validate senescence-associated phenotypes.

Regarding the in vivo/physiological context: it is probable that HDACi could trigger a rapid senescence-like response in other contexts or in vivo. Although mice could be treated with HDACi, this would be difficult to optimize and measuring senescence in tissues requires additional work

that goes beyond a single timely revision for this manuscript. Alternatively, we have now measured HDACi-induced senescence in multiple fibroblast cultures as well as in cancer cells. These results were added to this manuscript, particularly in Figure1 and Figure S1 to support the induction of the phenotype described here in a broader context)

We understand the important point of the reviewer to better understand the physiological role of the HDCAi-SASP, which we partly addressed in the previous reviews in one way suggested by the reviewer (adding more models). Overall, the scope of this paper is to describe the role of the non-canonical DDR for the SASP. To reach this point we used HDACi to trigger a SASP in absence of DNA damage and importantly we showed that the HDACi-SASP is highly similar to others SASP (XRA- REP)(see response to point 1 for more details). We also show that the HDACi-SASP occur in all cell models we tested including many independent normal cells and cancer cells that were added in the previous revision. At least 4 other groups have observed that HDACi induce senescence, so we can safely say that this phenotype if fully reproducible in multiple contexts (Ogryzko et al., 1996, Orjalo, Bhaumik et al., 2009, Pazolli et al., 2012, Pospelova, Demidenko et al., 2009). Now whether the HDACi-SASP occur *in vivo* or play a role in some context, this an interesting question. As an answer we can point to a previous paper that already demonstrated HDACi-induced senescent cells co-injected with cancer cell in xenografts stimulated cancer cell growth, as happens for other type of senescent cells induced by DNA damage (Pazolli et al., 2012). Overall there seem to be a relatively large amount of data supporting the idea that what we report with the HDACi-SASP is not an isolated artefact, we have now added a few sentences in the discussion of the paper to more clearly highlight the potential connections with cancer, particularly in the context where HDACi would be used to treat cancer.

To further follow-up on this question, the reviewer suggests strengthening the connection to NF-kB as a major transcription factor regulating the SASP. We probably did not emphasize previous knowledge regarding this connection appropriately. Would like to clarify that the NF-kB connection was previously described in multiple contexts including in the exact same cells and conditions used in this study (Chien, Scuoppo et al., 2011, Freund, Patil et al., 2011, Pazolli et al., 2012). An alternative to directly respond to the reviewer would be to validate the implication of p65 on the HDACi-SASP via an shRNA KD of the p65 subunit, but this experiment would not provide more information than already acquired previously following NaB treatment using a genetic inhibitor of the NF-kB pathway in the exact same cells we use ((Pazolli et al., 2012). We now make sure to correctly reference this specific point in the discussion of the manuscript.

To further follow-up on this question, the reviewer now suggests "In addition, authors could do co-culture experiment of NaB-treated cells with growing cells, and verify if cytokine secretion triggered by HDACi can induce senescence of growing cells in paracrine manner?". The paracrine effects of the SASP in general have been demonstrated many times including using the same cells we used in this study (Acosta, Banito et al., 2013, Borghesan, Fafian-Labora et al., 2019, Herranz, Gallage et al., 2015), and given that we have heavily validated that SASP profiles are similar between XRA, HDACi and even OIS there would be no reasons to doubt these paracrine experiments would yield the same results. An alternative to directly respond to the reviewer concerning paracrine HDACi SASP effects would be to validate the capacity of the HDACi-SASP to promote cancer cell proliferation and/or invasion, but we feel this experiment would not provide more information than already acquired previously using xenograft model to show that HDACi-induced senescent cells (same strain as we used) promote cancer cell growth (Pazolli et al., 2012). We now make sure to mention and reference this specific point in the discussion of the manuscript.

3. In Fig 3E, authors show reduction of SASP components following MRE11 depletion (shMRE11.5) compared to control (shGFP). However, the heatmap of immunoassay profiling doesn't seem to show such striking differences as it is quantified (on right). In addition, depletion of MRE11 without NaB treatment (- NaB), negatively affect the level of many cytokines. The explanation for the inconsistency is required.

We thank the reviewer for this observation. Indeed, this heat-map representation is not appropriate to visualize the differences of secretion between shGFP and shMRE11 following NaB treatment. We have re-formatted this data to better highlight the point. As noted by the reviewer, it is interesting to observe that the depletion of MRE11 on its own can reduce the secretion of certain factors. This reduction is also observed for mirin in figure 5G. This suggest that MRE11 could be involved in transcriptional regulation of a subset of SASP factors even in normal condition. We now comment on this observation in the results section.

4. Minor comment:

- Authors shall consider to be consistent with the figure units (unit of time), Fig 1E, 1F, 1G, 1H - units in days, Fig 1I units in hours

Thank to the reviewer for this observation. We changed the units in days in figure 1I

- In the text, authors refer to Fig. 3G (no such figure).
Thank to the reviewer for this observation. We changed the text fig3G to fig3E in the manuscript.

cross-comment referee #2:

The study is interesting and uncovers some novel angles are discussed in the official revision. The mechanistic part is fine. The study indeed lacks a bit of going beyond the standard thinking. Referee 1 thinks it could be further mechanistic studies the role of ATM in absence of DNA damage, which would be indeed exciting or connection to NFkB. I initially thought (and indicated in the first review) that the study lacks in vivo significance (or at least plausible explanation how the observed results can have specific physiological significance), in other words how we know that these tissue culture findings do happen in real life? This might be too much to ask for and I believe that each scientist should ask her/himself this kind of questions. Probably the authors could think of taking the study one step forward in either mechanistic or physiological direction. With this in mind the question raised by Referee 1 -is there enough novelty in the story to warrant publication at EBMO J ? seems relevant, while the study itself is solid.

With the overall consideration that the study is solid, and the additional efforts described above to answer comments from reviewer 2, we hope these cross-comments are solved for transfer to EMBO reports.

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1st Revision - Editorial Decision 24th Jun 2020

Dear Dr. Rodier,

Thank you for the submission of your revised manuscript. We have now received the enclosed report from referee 1 and I am happy to tell you that we can in principle accept your manuscript for publication here.

Only a few more changes will be required:

The source data for Fig 6C is labelled 6B, please correct.

Fig 6E source data the Nuclear soluble SF bands do not match the figure. Please correct.

Fig 6G source data the p65 red marker box is incorrect, it should also include the fourth band.

Fig EV5C source data the right hand panels are flipped, and MRE11 the red box is incorrectly placed. Please correct.

Some lanes in Fig 6 are still over-contrasted.

Please separate the conflict of interest statement from the authors contributions.

I would like to suggest a few minor changes to the title and abstract that needs to be written in present tense. Please let me know whether you agree with the following:

Non-canonical ATM/MRN activities temporally define the senescence secretory program

Senescent cells display senescence-associated (SA) phenotypic programs such as stable proliferation arrest (SAPA) and a secretory phenotype (SASP). Senescence-inducing persistent DNA double-strand breaks (pDSBs) cause an immediate DNA damage response (DDR) and SAPA, but the SASP requires days to develop. Here we show that following the immediate canonical DDR, a delayed chromatin accumulation of the ATM and MRN complexes coincides with the expression of SASP factors. Importantly, histone deacetylase inhibitors (HDACi) trigger SAPA and SASP in the absence of DNA damage. However, HDACi-induced SASP also requires ATM/MRN activities and causes their accumulation on chromatin, revealing a DNA damage-independent, non-canonical DDR activity that underlies SASP maturation. This non-canonical DDR is required for the recruitment of the transcription factor NF-κB on chromatin but not for its nuclear translocation. Non-canonical DDR further does not require ATM kinase activity, suggesting structural ATM functions. We propose that delayed chromatin recruitment of SASP modulators is the result of non-canonical DDR signaling that ensures SASP activation only in the context of senescence and not in response to transient DNA damage-induced proliferation arrest.

I will also send you a related manuscript file shortly with comments by our data editors that need to be addressed in the final manuscript file.

I look forward to seeing a final version of your manuscript as soon as possible.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors addressed the comments of the previous review process in satisfactory manner.

The authors addressed all minor editorial points.

2nd Revision - Editorial Decision 16th Jul 2020

Dr. Francis Rodier
Universite de Montreal
Radiologie, radio-oncologie et mé
Room Y-4621-1
2099 Alexandre DeSeve
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Canada

Dear Dr. Rodier,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Esther Schnapp, PhD
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The data shown in figures should satisfy the following conditions:

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- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We choose to perform each experimental condition triplicate or more and reproduce the experiment independently at least 2 (for supplementary data) or 3 times.
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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	The variances are similar

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We provide a list of antibodies used in supplementary table 1
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of primary cells or cell linesis described in the Material and method section

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Sequences of shRNA were given in the Material and method section
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA